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Seasonal dynamics and potential drivers of ranavirus epidemics in wood frog populations

Emily M. Hall[1,3] · C. S. Goldberg[2] · J. L. Brunner[1] · E. J. Crespi[1]

Abstract
Epidemics in wildlife populations often display a striking seasonality. Ranaviruses can cause rapid, synchronous mass mortality events in populations of wood frog (*Rana sylvatica*) larvae in the summer. While there are several possible explanations for this pattern—from seasonal introductions of the virus to environmental stressors to windows of susceptibility to mortality from infection during development—most studies have focused on single factors in laboratory settings. We characterized the time course of ranavirus epidemics in eight ephemeral ponds in Connecticut, USA, measuring the prevalence and intensity of infections in wood frog larvae and *Ranavirus* DNA in water samples using environmental DNA methods. We found little evidence that the timing of pathogen introduction affected the timing of epidemics (rising prevalence) or the resulting die-offs. Instead, we observed a pulse in transmission asynchronous with die-offs; prevalence reached high levels ($\geq 50\%$) up to 6 weeks before mortality was observed, suggesting that die-offs may be uncoupled from this pulse in transmission. Rather, mortality occurred when larvae reached later stages of development (hind limb formation) and coinciding water temperatures rose ($\geq 15\,^{\circ}\mathrm{C}$), both of which independently increase pathogenicity (i.e., probability of host mortality) of infections in laboratory experiments. In summary, the strong seasonality of die-offs appears to be driven by development- and/or temperature-dependent changes in pathogenicity rather than occurring chronologically with pathogen introduction, after a pulse in transmission, or when susceptible host densities are greatest. Furthermore, our study illustrates the potential for eDNA methods to provide valuable insight in aquatic host–pathogen systems.

Keywords Seasonal epidemiology · Ranavirus · Amphibian · Disease susceptibility · Environmental DNA

Communicated by Pieter Johnson.

✉ Emily M. Hall
   emily.m.hall@vanderbilt.edu

1   Washington State University, School of Biological Sciences, P.O. Box 644236, Pullman, WA 99164-4236, USA

2   Washington State University, School of the Environment, P.O. Box 646410, Pullman, WA 99164-2812, USA

3   Present Address: Department of Pathology, Microbiology, and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA

Introduction

Infectious disease outbreaks are often conspicuously seasonal, with greater incidence of infection or mortality occurring at predictable times of year (Altizer et al. 2013; Dowell 2001; Grassly and Fraser 2006). Seasonal pulses in the density of susceptible individuals due to reproduction, breeding aggregations, or increases in resources, are common explanations for seasonal epidemics in natural populations (White et al. 1996), but environmental forces affecting host infectiousness (e.g., pathogen shedding rates) or susceptibility (i.e., probability a host becomes infected) could also be important (Langwig et al. 2015; Nelson et al. 2002). Disentangling the complex mechanisms that drive seasonality in host–parasite interactions remains a challenge for ecologists (reviewed in Altizer et al. 2006), but one that is increasingly relevant in the face of global change.

To better understand seasonal epidemics, we studied the natural course of ranavirus-related die-offs in wood frog

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(*Rana sylvatica*) populations in ephemeral ponds. Ranaviruses (family *Iridoviridae*) are emerging, lethal viruses of ectothermic vertebrates around the world (Duffus et al. 2015). Among amphibians, wood frogs are highly susceptible to ranavirus infections and mortality (Brand et al. 2016; Haislip et al. 2011). Ranavirus-related mortality events often exhibit a detectable seasonality in aquatic amphibian larvae (Green et al. 2002); die-offs of wood frog larvae occur during mid-summer in the Northeast USA (Gahl and Calhoun 2008; Green et al. 2002). Seasonal epidemics in many systems are related to a pulse in host reproduction increasing the density of susceptible individuals in the population (Wells et al. 2015; White et al. 1996). In the case of wood frogs, there is a clear pulse in susceptible hosts when larvae hatch *en masse* in early spring, but while recently hatched larvae are susceptible to ranavirus infections (Haislip et al. 2011), die-offs are typically observed in summer months (Green et al. 2002). While this pulse of susceptible individuals does not seem to be a primary driver of the timing of dieoffs, other putative, non-mutually exclusive mechanisms of seasonality might be involved including the seasonal introduction of ranaviruses or changes in transmission (i.e., force of infection or susceptibility to infection) or pathogenicity (i.e., mortality of an infected host).

Many seasonal epidemics are associated with the timing of the introduction of a pathogen into a host population (Grassly and Fraser 2006). Potentially, die-offs are not observed in spring when host density is highest due to an absence of the pathogen in this ephemeral system. The source and timing of introduction is unknown, but ranaviruses likely do not persist from the previous year in dried sediment as they are inactivated when dried (Brunner et al. 2007) and degrade rapidly in pond water, at least at warmer temperatures (Johnson and Brunner 2014; Nazir et al. 2012). Rather, hatchlings are likely exposed to ranaviruses after sub-lethally infected wood frog adults (Crespi et al. 2015) or other reservoir species contaminate pond water (Duffus et al. 2008). If the introduction of ranaviruses into the system is driving the timing of die-offs, we would expect the first detection in pond water to be followed by increases in prevalence in larvae, based on laboratory experiments in which infections are transmitted rapidly (Brunner et al. 2017; Harp and Petranka 2006) and are typically lethal within 1–2 weeks (Haislip et al. 2011).

The seasonality of epidemics and die-offs might also be attributed to changes in transmission and pathogenicity of ranavirus infections. Viral replication rates in cell culture are temperature and strain dependent (Ariel et al. 2009; Chinchar 2002), as are immune responses of ectothermic vertebrates, thus predicting the net effect of temperature on the probability of infection and mortality is challenging (Brunner et al. 2015). However, two laboratory experiments have found that warmer temperatures (10 vs 25 °C and 14 vs 22 °C, respectively; Brand et al. 2016; Echaubard et al. 2014) led to greater mortality in ranavirus-infected tadpoles. Exposure to ranavirus during developmental stages in late limb bud formation (at constant temperatures) also led to more severe infections (Warne et al. 2011; but see Haislip et al. 2011). During these stages, ranavirus infection induces an increase in corticosterone (Warne et al. 2011), a hormone that suppresses splenocyte proliferation in infected wood frogs (Kirschman et al. 2018), and at high levels is generally immunosuppressive across vertebrates (Dhabhar 2009; Rollins-Smith 1998). While these studies suggest temperature and developmental stage affect susceptibility to mortality from infection, little is known about how these factors affect disease-related mortality in natural populations or how they affect other aspects of disease dynamics such as transmission rates throughout ranavirus epidemics.

Here, we measured host density, ranavirus prevalence and intensity of infection from the time of breeding until metamorphosis in eight ephemeral ponds in Northeastern USA, to determine the relationships between seasonal changes in environmental and developmental factors with the progression of epidemics in natural populations. We focused on ephemeral pools because we previously observed seasonal die-offs of wood frog larvae, they are abundant on the landscape, and communities are typically dominated by the larvae of one or two amphibian species. We used an environmental DNA (eDNA) approach to measure *Ranavirus* DNA shed by the community of hosts in the pond, as we found that eDNA concentrations were highly correlated with viral titers found in wood frog larvae and coincided with the timing die-offs (Hall et al. 2016). Because the prior study sampled ponds only twice throughout the season (in 20 ponds), the goal of this study was to describe a more fine-scale timecourse of ranavirus epidemics and further validate previous findings. Given that waterborne active virus can be a route of infection (Robert et al. 2011) and eDNA levels were shown to be related to the probability larvae will become infected in the laboratory (Araujo et al. 2016), eDNA titers could reflect the force of infection (the rate at which susceptible individuals acquire new infections). By measuring the change in eDNA and the density of infected individuals over the course of epidemics, we can infer whether transmission rises at a constant rate after the initial introduction. Transmission rates have yet to be rigorously examined in natural populations (except in one pond; Todd-Thompson 2010), and measuring both eDNA and larval titers over time can determine whether epidemics are consistent with a simple epidemic model (e.g., an SI model) or vary seasonally. Although correlative in nature, this study is among the first to rigorously document natural ranavirus epidemics to validate hypotheses derived from laboratory experiments.

Methods

Site description

In 2014, we monitored eight ephemeral, fishless wetlands dominated by wood frogs in mixed hardwood forests within and near Yale Myers Forest in northeastern Connecticut, USA. These seasonal depressional wetlands completely dry by the middle or end of summer each year and fill with autumn rains. Wood frogs are explosive breeders that arrive first after snow melt. Breeding occurs within a few days and then adults disperse into the surrounding terrestrial habitat. *Ambystoma maculatum* breed in these sites around the same time as wood frogs. Amphibian species that may breed later in the season if the hydroperiod is longer include *Notophthalmus viridescens, Pseudacris crucifer,* and *Hyla versicolor. Ambystoma opacum* breed in autumn and can overwinter as larvae under the ice. Adult *Rana catesbieanus* and *R. clamitans* have been observed but do not use these ephemeral sites for breeding. Because these species are known hosts of ranaviruses, we recorded observations during diurnal visits. We chose sites where wood frogs were by far the most abundant species (93% of the larvae caught were wood frogs in our study the previous year) and at varying distance from paved roads. In the previous study, three of the eight ponds had ranavirus-related die-offs and we detected ranaviruses in both eDNA and larval wood frog tissue samples in all eight ponds (Hall et al. 2016).

Surveillance methods

We collected three eDNA samples, one water sample for biogeochemical analysis, and 10 live wood frog larvae (if present) via dip-netting each sampling date, which occurred every 2 weeks from the beginning of ice-off (31-Mar-2014) to 15-Jul-2014, at which point ponds had dried or nearly dried and larvae were no longer present. All waders, gear, and nets were bleached between sites by soaking in 10% bleach solution for several minutes to prevent contamination and rinsed with tap water. Environmental DNA sampling occurred as described in Hall et al. (2016). Briefly, before entry into the pond, surface water was sampled at three locations roughly 90° apart. At each point on the shore, 250 ml of pond water was scooped with a new, clean disposable cup into a 250 ml 0.22 μm nitrocellulose filter (Nalgene Analytical Filter funnels, ThermoScientific, Waltham, MA, USA) on a vacuum flask and hand pumped dry. With a clean glove, the filter was folded and placed in 1 ml of 100% ethanol. We also filtered distilled bottled water according to the same protocol as a field negative

alongside the three eDNA samples. We then collected a 150 ml of water sample at 10 cm depth filtered through a 0.45-μm syringe filter and kept frozen for biogeochemical analysis (see below). Ten wood frog larvae were selected haphazardly from a transect through the pond. They were euthanized with an overdose of benzocaine (0.1%) and preserved individually in 10 ml of 100% ethanol and later measured snout–vent length (SVL) and staged according to Gosner (1960). We also measured conductivity, pH, and temperature at 10 cm below the surface using a water quality meter (YSI Professional Plus Handheld Multiparameter Water Quality Meter, YSI Inc., Yellow Springs, OH, USA) at the deepest point (or at a depth of ca. 1 m if depth exceeded 1 m) in the pond. Every other week post swimming stage, we performed time-limited dip-net surveys based on area of the pond, and counted wood frog and *Ambystoma* larvae and carcasses separately to estimate density (per m^2) following Werner et al. (2007).

We visited the ponds every week to monitor for mortality by walking the perimeter for a visual encounter survey of carcasses (recommended technique; Gray et al. 2015). We considered the start of a die-off event the first day that five or more amphibian carcasses were observed. These events were characterized by > 95% mortality within 1–2 weeks. We observed carcasses for 1–2 weeks in previous mortality events in this system of ponds (Hall et al. 2016) and given the small size and shallow depth of these pools, we had a high confidence of observing die-offs; however, mortality occurring at low frequency or early during the larval period may not be detected by this method. After a die-off was observed, we adjusted sampling eDNA and larvae to every week thereafter until the pond dried or tadpole carcasses were no longer found.

DNA extractions and qPCR

DNA from filters was extracted using the Qiashredder and DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) method of Goldberg et al. (2011) in a laboratory dedicated to low-quantity DNA samples. Liver tissue was dissected from each larva in a separate laboratory with tools that were flamed between samples. DNA was then extracted from these samples using the tissue protocol of the Qiagen DNeasy Blood & Tissue kit. Extraction batches of each sample type included extraction negatives of buffers in which sterilized tools were immersed, all of which were negative for amplification.

To calculate concentrations of *Ranavirus* DNA, we used a Taqman real-time PCR assay that amplifies a 96 bp region of the major capsid protein in all known ranaviruses except for the Santee-Cooper lineage that infects fish and does not amplify members of other genera in Iridoviridae, including Lymphocystivirus and Megalocytivirus (Stilwell et al. 2018). All samples were run in triplicate 20 μl PCR reactions

for 45 cycles on a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA). Each reaction had 5 µl of template DNA, which had been diluted to 20 ng of DNA/µl (measured with NanoDrop-2000; Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's suggestions. Each plate included a standard dilution series, from 5×10^2 to 5×10^6 copies of a synthetic gBlock (IDT, Coralville, IA) gene fragments of the target region to provide a standard curve and a water-only no template control as a negative control. eDNA samples were run with one well containing TaqMan® Exogenous Internal Positive Control (Thermo Fisher Scientific, Waltham, MA) to test for PCR inhibition. Samples showing signs of PCR inhibition were cleaned using the OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA); however, four eDNA samples remained inhibited and were removed from analyses. Samples for which two or three wells showed clear amplification were scored as positive, those with no amplification were negative. Inconsistent samples (1 well testing positive) were rerun and if at least one well amplified on the second run, the sample was scored as positive. Samples with a coefficient of variation > 15% between triplicates were also rerun. Viral quantities for positive eDNA samples were averaged across wells and back-transformed to the mean $\log_{10}$ copies per filtered volume or per sample for tissue.

Biogeochemical analysis

Filtered water samples were analyzed for total nitrogen (total Kjeldahl nitrogen; the sum of organic and ammonia nitrogen), and total phosphorus (by persulfate digestion) concentrations, according to standard methods (APHA 1998) on a SmartChem 200 discrete analyzer (Westco Scientific Instruments, Brookfield, CT). Filtered water samples were analyzed for chloride concentrations by ion chromatography on a Dionex ICS-1100 (Thermo Fisher Scientific, Waltham, MA).

Statistical analysis

We conducted analyses using R version 3.2.3 (R Core Team 2015). To determine the relationship between average eDNA and larval titers at each visit (excluding individuals or filters with no detectable amplification) we used a linear mixed model with visit date and pond as random effects (*lmer* function in the *lmerTest* package; Kuznetsova et al. 2016). We examined whether other factors (i.e., tadpole density, developmental stage, temperature, and conductivity) affected the relationship between eDNA and larval titers by looking for interactions in mixed models (*lmer* function). We also examined whether these same factors affected the relationship between prevalence and larval titer in logistic mixed models with visit date and pond as random effects (function glmer, package lme4, binomial family, logit link; Bates et al. 2015). To examine whether transmission occurred at a constant rate, we compared the timing associated with the introduction of ranaviruses, the timing of when prevalence reached particular thresholds, as well as the rate at which the density of infected individuals (prevalence × tadpole density) increased through time. We used logistic mixed models to evaluate the effect of several biological and environmental factors on the probability of infection and observed mortality (die-off = 1, no die-off = 0) with visit date and pond as random effects (function glmer, package lme4, binomial family, logit link; Bates et al. 2015). We chose temperature, percent of original depth, chloride concentration (an environmental stressor of wood frog larvae; Hall et al. 2017), and larval developmental stage and snout–vent length (SVL) as a priori predictors of mortality and prevalence, but also explored additional variables to better characterize pond conditions (pH, total nitrogen, and total phosphorous). We examined the correlations between environmental factors using Pearson's r (Table 1). When logistic models would not converge due to complete or quasi separation of responses, we used the bias reduction function in the package *brglm* (Kosmidis 2013).

Table 1 Matrix of Pearson's correlation coefficients between variables that were related to mortality in logistic regressions (Fig. 3)

	Temperature (°C)	Larval titer (copies)	eDNA titer (copies/ml)	Prevalence	Developmental stage	Visit date
Temperature (°C)	1					
Larval titer (copies)	0.724	1				
eDNA titer (copies/ml)	0.755	0.882	1			
Prevalence	0.779	0.885	0.790	1		
Developmental stage	0.888	0.819	0.855	0.778	1	
Visit date	0.927	0.819	0.808	0.794	0.952	1

Titers and developmental stages were averaged across samples ($n = 3$ eDNA; $n = 10$ larvae). Larval and eDNA ranavirus titers were Log_{10} transformed. Prevalence represents the proportion infected out of the total larvae collected at each visit

Results

In five of the eight ponds surveyed, at least one of the three eDNA samples was positive at the time eggs were laid (two ponds had 3/3 positive samples), and two ponds had positive eDNA samples prior to immigration of any spring breeding amphibian species, although titers were very low (~ 1 copy/ml). Low-level ranavirus infections were detected in larvae as early as the hatchling stage (Gosner stage 20). By the end of the season, four of eight ponds experienced die-offs (when we found 5–50 carcasses during dip-net surveys) within a 2-week window (10-Jun-2014 to 25-Jun-2014), three of which had die-offs in the previous year (Hall et al. 2016). The other four ponds dried between June 18 and 24, before larvae metamorphosed or mortality was observed. Although the density of *Ambystoma* larvae early in the season was < 5% of the total larval density, a few *Ambystoma* carcasses (as well as living larvae) were observed during wood frog die-offs. The majority (92%) of larvae caught in dip-net surveys were wood frogs.

Prior to die-offs or rapid pond drying, developmental rates and the water temperature increases were similar across ponds (Fig. 2); however, as water levels dropped, tadpole density increased in the four drying ponds and decreased in the four die-off ponds. Within the ponds with die-offs, the change in the log density of infected larvae was variable and did not increase over time as the null model would predict given constant transmission (Electronic Supplemental Fig. 1). Further, the log density of infected larvae was not associated with the increases in eDNA ($t_{16.54} = 0.162$, $P = 0.87$) or larval titers ($t_{19.14} = 0.336$, $P = 0.74$).

The time between when ranaviruses was first detected in water or larvae, or when prevalence reached particular thresholds (e.g., $\geq 25\%$, which we should be able to detect with ~ 95% probability given a sample of ten larvae) and when die-offs occurred varied among the four ponds (Figs. 1, 2). For instance, one pond reached 50% prevalence 6 weeks before the die-off was observed, and another did not reach this mark until the visit when the die-off began (Fig. 2). Infection prevalence saturated above 90% prior to when larval titers reached their maximum in three of these four ponds (Figs. 1, 2); in the other pond, which experienced a die-off 2 weeks earlier, the peak in prevalence and larval and water titers occurred simultaneously (Figs. 1, 2). Note in two cases, eDNA titers increased before larval titers (see Fig. 1). Further, the time from first detection to 100% prevalence varied from 4 to 8 weeks across epidemics (Fig. 2). Prevalence increased as infection intensity rose in larvae ($n = 45$ visits; average of 10 larvae except after a die-off when fewer larvae were
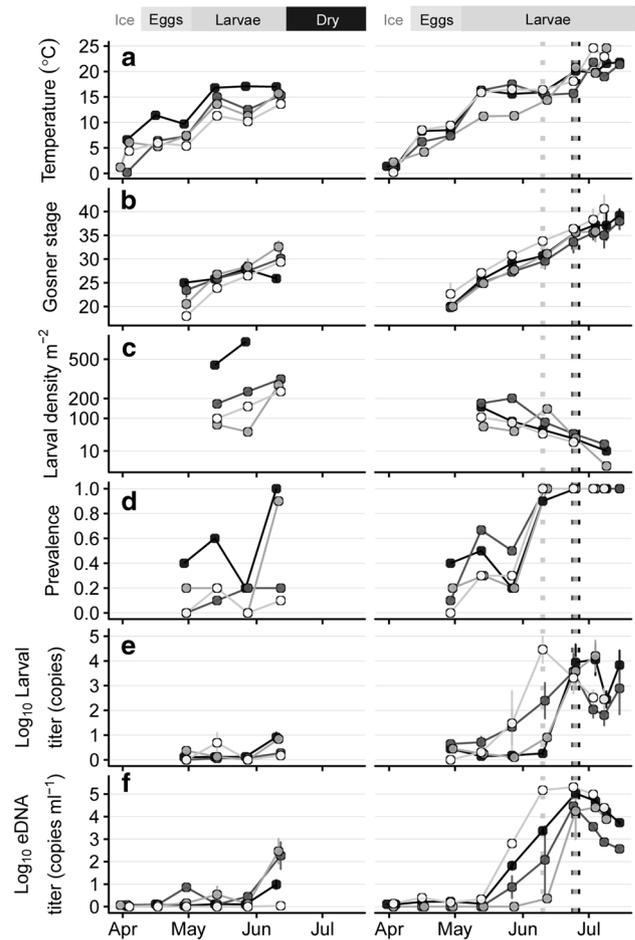

Fig. 1 Temporal dynamics of ranavirus epidemics of wood frogs (*Rana sylvatica*) in ephemeral ponds. **a** Surface water temperature (°C), **b** mean ($\pm$ SD) developmental stage (Gosner 1960), **c** wood frog density (larvae/m²), **d** prevalence of ranavirus infection (proportion positive in qPCR; $n = 45$ visits when larvae were present, 4–10 larvae/visit) of wood frog larvae, **e** mean ($\pm$ SE) titer in liver of infected wood frog larvae, **f** mean ($\pm$ SE) *Ranavirus* eDNA titer ($n = 64$ visits, 3 water samples/visit) in eight ephemeral ponds surveyed in this study. Four ponds dried before metamorphosis occurred (left), and the other four ponds had ranavirus-related die-offs (right). The timeline above panels shows the breeding and larval period of wood frogs and when ponds dried on the left. Observations of the first carcasses, the start of a massive die-off event is indicated by vertical dotted lines. Each pond is represented by a unique shade of gray in each panel. For graphing, we added 1 to titer averages before log transforming

found; $\beta_{\text{Log larval titer}} = 2.608 \pm 0.969$, $z = 2.692$, $P = 0.007$), and there were no significant interactions that affected this relationship (*R. sylvatica* larval density: $z = -0.124$, $P = 0.90$; Gosner stage: $z = -0.171$, $P = 0.86$; temperature: $z = -0.707$, $P = 0.48$; chloride: $z = 0.279$, $P = 0.78$; pH $z = -0.411$, $P = 0.68$; total organic nitrogen: $z = 0.148$, $P = 0.88$, total phosphorus: $z = 0.900$, $P = 0.37$).

In ponds with die-offs, both larvae and eDNA titers remained low (< 8 copies per sample or ml) for several
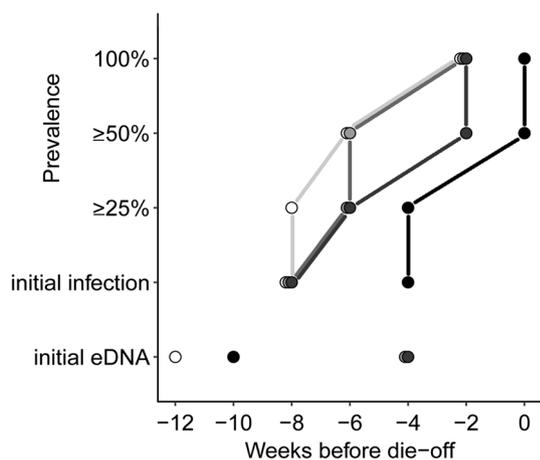
Fig. 2 Timing of ranavirus introduction and subsequent increase in prevalence of infection in the four study ponds with die-offs. Although breeding occurred within 4 days across ponds, there appears to be differences in time between when ranaviruses were first detected in eDNA samples ("initial eDNA") and larval tissue ("initial infection"), and when prevalence reached 25, 50, and 100% in samples of wood frog larvae (y-axis). The timing between an increase in prevalence (percentages on the y-axis) and when the die-off occurred also differed across ponds. Each shade of gray corresponds to each pond in the right panel of Fig. 1

weeks after hatching, but then increased rapidly in early June (Fig. 1, right panels). The timing between when ranaviruses was first detected and the first observation of mortality varied between 4 and 8 weeks in larvae and 4–12 weeks in water (Figs. 1, 2). The peak in eDNA and larval titer occurred at the time of mortality, but the rate of increase varied among the four ponds. In three ponds, titers began to rise 6 weeks prior to a die-off, but only 2 weeks prior in the fourth pond, which experienced a die-off 2 weeks earlier than the others (Figs. 1, 2). The timing of die-offs did not seem to be related to the initial population size (based on egg mass counts), as the earlier die-off pond had 182 egg masses and the other three ranged from 81 to 347 egg masses.

Unlike the ponds with longer hydroperiods, the four ponds that dried in mid-June maintained low titers (< 3 copies/ml) and prevalence less than 25% (in three of four ponds) through the end of May (Fig. 1, left panels). We observed two different patterns during the last visit in early June: two ponds maintained low prevalence and titers until they dried, and the other two had increased prevalence and titers in water, but minimal increases in larval titer (Fig. 1). Individuals were on average Gosner stage 30 ± 1 (prior to toe development) and no froglets were observed at the last time point before these ponds dried.

Of the suite of environmental factors measured, only water temperature had a strong relationship with ranavirus prevalence and timing of mass mortality (Fig. 3a, c). The odds of infection increased about 2.5-fold with

every 1 °C increase in pond temperature ($n = 45$ visits; $\beta_{\text{Temperature}} = 0.899 \pm 0.358$, $z = 2.509$, $P = 0.012$), with a predicted probability of infection of 95% at 21 ± 2 °C. The odds of a mass mortality event increased even more sharply with temperature, about 2.9-fold with every 1 °C ($\beta_{\text{Temperature}} = 1.069 \pm 0.372$, $z = 2.871$, $P = 0.0041$); there was a 95% probability of a die-off predicted at 20 ± 1 °C. When die-offs and the peak in titers were observed, water temperatures ranged from 15.7 to 20.8 °C (Fig. 3e). Generally, temperatures were around 15 °C 4–6 weeks prior to a die-off, except in one pond where the temperature leveled out at 11 °C and then warmed rapidly which corresponded to when the eDNA titer of that pond sharply increased (Fig. 1). There was a slight trend of increasing odds of a mortality event with increasing chloride concentration ($\beta_{\text{Chloride}} = 0.044 \pm 0.025$, $z = 1.769$, $P = 0.077$), note that three of the four ponds experiencing die-off were near roads where road salt is applied (Electronic Supplemental Table 1). Die-offs were not related to declining water depth (as measured by the percent of initial pond depth; $z = 1.097$, $P = 0.27$) or several other factors (pH $z = -0.536$, $P = 0.59$; total organic nitrogen, $z = 0.649$, $P = 0.52$; total phosphorus, $z = 0.203$, $P = 0.84$).

Larval developmental stage, which was highly correlated with temperature (Table 1), also had a strong relationship with prevalence and probability of a mortality event (Fig. 3b, d). The odds of infection and mortality increased 1.7- and 3.4-folds with each Gosner stage, respectively ($n = 45$ visits; $\beta_{\text{Gosner stage}} = 0.527 \pm 0.167$, $z = 3.157$, $P = 0.0016$; $\beta_{\text{Gosner stage}} = 1.213 \pm 0.494$, $z = 2.457$, $P = 0.014$). The average developmental stage when the die-off and a peak in titer was observed ranged from Gosner 34–36 (Fig. 3f). There was a 95% chance of infection and mortality at Gosner stage 37 ± 3 and 35 ± 1, respectively. The odds of infection and mortality also increased with average body size ($\beta_{\text{SVL}} = 0.613 \pm 0.203$, $z = 3.012$, $P = 0.0026$; $\beta_{\text{SVL}} = 1.198 \pm 0.434$, $z = 2.763$, $P = 0.0057$), which was correlated with average developmental stage ($r = 0.925$).

Concentrations of *Ranavirus* DNA in the pond water (reflecting the community of hosts; $n = 45$ visits) was strongly related to the titer in larval tissues across visits in which both samples were collected ($\beta_{\text{Log larval titer}} = 0.970 \pm 0.105$, $t_{19.647} = 9.246$, $P < 0.001$). Environmental DNA methods detected ranaviruses in 88.9% (40/45) of the visits in which at least one larva was infected. There were a few visits in which eDNA detected ranaviruses when the larvae collected were negative, but these incongruencies occurred when titers were very low (< 3 copies). When we examined whether factors affected the relationship between larval and eDNA titers, we did not find any significant interactions (*R. sylvatica* larval density: $t_{24.89} = -0.985$, $P = 0.33$; Gosner stage: $t_{38.36} = -0.179$, $P = 0.85$; temperature: $t_{22.56} = 0.307$, $P = 0.76$; pH: $t_{20.33} = -0.177$, $P = 0.86$;

Fig. 3 The relationship between (**a**, **b**) prevalence, (**c**, **d**) probability of mortality, or (**e**, **f**) average $\log_{10}$ larval ranavirus titer by either (left) temperature or (right) average developmental stage (Gosner 1960). Lines represent the logistic fit and shading represents the 95% confidence interval in panels a through d (loess lines are depicted in **e**, **f**). Each point represents a single pond visit (6–11 visits at 8 ponds, $n = 45$). The odds of infection and a mortality event increased with temperature (Generalized linear model; $\beta_{\text{Temperature}} = 0.899 \pm 0.358$, $z = 2.509$, $P = 0.012$; $\beta_{\text{Temperature}} = 1.069 \pm 0.372$, $z = 2.871$, $P = 0.0041$). The odds of infection and a mortality event increased with developmental stage (generalized linear model; $\beta_{\text{Gosner stage}} = 0.527 \pm 0.167$, $z = 3.157$, $P = 0.0016$; $\beta_{\text{Gosner stage}} = 1.213 \pm 0.494$, $z = 2.457$, $P = 0.014$)
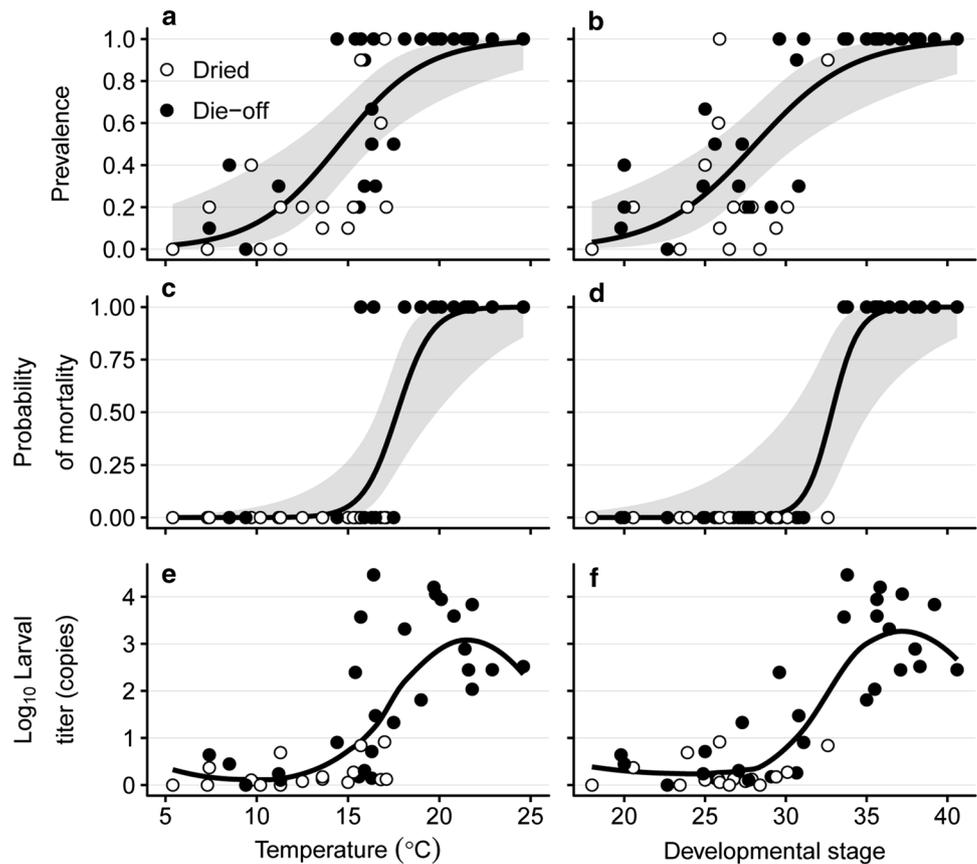
chloride: $t_{0.31} = 0.011$, $P = 0.99$; total organic nitrogen: $t_{6.24} = 0.735$, $P = 0.49$; total phosphorus: $t_{27.58} = 0.382$, $P = 0.71$).

Discussion

The seasonal dynamics exhibited in many host–parasite systems are driven, directly or indirectly, by changing environmental conditions, and understanding these drivers can provide crucial estimates for modelling epidemiology under climate change scenarios (Altizer et al. 2006, 2013). Our study of the temporal dynamics of multiple wood frog-ranavirus epidemics suggests that seasonal die-offs occur in relation to greater host susceptibility. Both rising temperature and development stage were highly correlated with increases in prevalence and transmission, although attaining a critical developmental stage (hind-limb development) was more closely associated with timing of die-offs. By contrast, the timing of pathogen introduction, a change in transmission, or the density of susceptible or infected individuals did not strongly coincide with seasonal dynamics of ranavirus epidemics. Here, we discuss the hypotheses generated from our observations as groundwork for future mechanistic research on forces that affect host susceptibility and seasonal die-offs.

The first detection of *Ranavirus* DNA (~1 copy/ml) at the end of winter in two ponds suggests an environmental reservoir between epidemics. Both sites had positive eDNA samples in the previous year and one exhibited a die-off of wood frog larvae. *Ranavirus* spp. can retain infectiousness at 4 °C or while frozen for months under semi-natural experimental conditions (Munro et al. 2016; Nazir et al. 2012) and most of the surface water was frozen during the first visit. However, we do not know if the *Ranavirus* DNA comes from infectious virions and we cannot exclude the possibility that some infected adults migrated to the ponds prior to sampling and were missed in surveys. The initial detection in both water and wood frog larvae did not coincide with the breeding of spotted salamanders (*A. maculatum*) since they bred 2–3 weeks after the wood frogs this year. Marbled salamanders (*A. opacum*) breed in the fall and larvae could serve as an over-wintering host, although, they are not highly susceptible to infection in laboratory exposures (Hoverman et al. 2011). Additional field observations are needed to identify the initial source(s) and the means of year-to-year persistence in this system.

Given our estimate of pathogen introduction, we observed a pulse in transmission—as evidenced by increases in the prevalence of infection and eDNA levels in May—which occurred asynchronously and long after initial exposure.

This finding was not consistent with our expectation that prevalence would reach 100% within 1–2 weeks from ranavirus introduction, based on estimates of transmission rates in mesocosm experiments (Brunner et al. 2017; Greer et al. 2008). After 4–6 weeks, prevalence rose dramatically in six of the eight ponds, reaching 100% typically 2 weeks before mortality was observed. The density of infected individuals, however, did not rise at a constant exponential rate, suggesting a change in transmission rate (Electronic Supplemental Fig. 1). The apparent surge in transmission was likely not caused by a decrease in resistance to infection, as wood frogs larvae are equally likely to become infected across stages (Haislip et al. 2011; Warne et al. 2011) and there is little evidence to suggest that temperature affects the probability an individual becomes infected with a *Ranavirus* (reviewed in Brunner et al. 2015). Because titers in larvae and eDNA increased during this time, we suspect that as larvae develop more intense infections later in the season, they also become more infectious, as larvae that shed more virions infect a greater number of naïve individuals (Araujo et al. 2016). These findings pose the question of whether host physiology and/or viral replication rates explain the non-constant rate of transmission.

Also counter to our expectations, ranavirus-related mortality events were temporally uncoupled from the initial rise in prevalence. Prevalence began to rise to ~50% in our study ponds about 4–6 weeks before die-offs, when titers were below 2 copies per liver sample or ml of water and did not reach levels above 100 copies until 2–4 weeks thereafter. Experimental infections with high doses (10^3 pfu/ml) typically lead to 100% infection and mortality within 7–10 days (Duffus et al. 2008; Warne et al. 2011); so the apparent delay in mortality suggests some other factors strongly affect the outcome of infections. One possibility is a critical window of susceptibility in development, as mortality from ranavirus infection varies with developmental stage for wood frogs and other species of amphibians (Haislip et al. 2011; Warne et al. 2011) as well as other amphibian host–parasite systems (e.g., to malformations and mortality from trematodes; Johnson et al. 2011; Kelly et al. 2010). Although we likely missed low levels of mortality in early stages when larvae are small, the developmental stages during which mortality was first observed (Gosner 33–35) were consistent with those stages at which experimental infections became notably more lethal (Warne et al. 2011), even though the time when larvae reached these stages varied among populations.

Because viral replication and amphibian developmental rates are both temperature-dependent (Chinchar 2002; Denver et al. 2002; Hayes et al. 1993), temperature likely plays both direct and indirect roles in the seasonality of ranavirus epidemics. Ranaviruses generally replicate faster in cell culture at warmer temperatures (between 12 and 32 °C; Chinchar 2002), although optimal ranges depend on typical host body temperatures (Ariel et al. 2009). Host immune responses are also temperature dependent, thus predicting the net effect of temperature on the outcome of infections is difficult a priori (reviewed in Brunner et al. 2015), but larval mortality from infection is generally more rapid at higher temperatures (above 20 °C, Brand et al. 2016; Echaubard et al. 2014). Brand et al. (2016) found that ~100% of wood frog larvae exposed at Gosner stage 30 died within 10 days after the holding temperature was increased from 10 to 12 °C, whereas there was no mortality in the previous 4 weeks, suggesting even a small temperature increase might change sublethal infections to lethal ones. In our surveys, however, temperature increases did not coincide with the rise in prevalence or the timing of die-offs: temperatures remained constant for 4–6 weeks prior to die-offs in three of the four die-off ponds and increased by 6 °C 2 weeks prior to a die-off in the other. Moreover, if ~12 °C is an important threshold, we would have expected to see die-offs 4–6 weeks earlier than observed. Water surface temperatures were above 10 °C in early to mid-May, when tadpoles were still between Gosner stages 24–27 and larval titers were still below ~1 copy/sample. Over the next 2 weeks, temperatures rose to around 15 °C, which corresponded with a modest rise in larval titers (<50 copies/sample) and an increase in prevalence, but the first signs of mortality did not occur for another 4–6 weeks. While we did not find a clear threshold water temperature or changes in temperature associated with mortality events, there is ample evidence to suggest that temperature plays an important role in ranavirus–host interactions directly (e.g., viral replication rates) and indirectly (e.g., host development rates, immune function). Moreover, it is also possibly correlated changes to the physical environment not measured here (e.g., reduced dissolved oxygen; Moore and Townsend 1998) could contribute to stress and increase the probability of death. Thus, while our study provides ecological context for these laboratory experiments, further research is needed to differentiate the unique and combined effects of temperature and developmental rate on transmission and the probability of mortality.

The use of eDNA sampling allowed us to expand the study of aquatic disease dynamics in time and scope in natural populations, well beyond the typical application of identifying and surveying aquatic parasite communities (Bass et al. 2015). We were able to estimate the timing of introduction of ranaviruses into ponds, measure *Ranavirus* DNA in ponds after carcasses degraded, and determine that transmission is not constant in wood frog-ranavirus epidemics but appears to accelerate. We further confirmed that eDNA levels correlate strongly with the intensity of infection in wood frog tadpoles, the most abundant and susceptible host (Hall et al. 2016); but the relationship between eDNA titers and prevalence or force of infection was weak, suggesting a more complex interaction. In

terms of predicting the potential risk for disease impact on amphibian communities, multiple visits are required to capture the rapid increase in viral titer that characterizes an epidemic and should accompany sampling tissues from target species and carcasses.

We began this study with several possible mechanisms found to influence mortality from ranavirus infection in laboratory exposures. Our observations suggest that there are seasonal factors that alter pathogenicity—leading to more intense and transmissible infections—and these changes can be a potential dominating force in seasonal epidemics. Moreover, while there are multiple proximate mechanisms that may lead to increased susceptibility, we posit that developmental stage and rising temperatures, or the interaction of the two, are most important. Because summer temperatures are predicted to rise in the Northeast (Hayhoe et al. 2008), phenology and developmental rates of amphibians, and ranavirus replication and transmission dynamics, are likely to be affected by climate change. Likewise, the potential for road salt runoff to affect ranavirus disease dynamics needs further investigation, since three of the four ponds with die-offs had elevated chloride levels. Perhaps the most interesting and difficult challenge in this system will be characterizing the feedback between increasing transmission and pathogenicity. Environmentally driven changes in pathogenicity are often considered independently of transmission (but see Lafferty and Holt 2003), but these findings suggest that these are intimately linked.

Author contribution statement EMH, CSG, JLB, and EJC designed the experiment. EMH performed field work and processed samples. EMH and JLB analyzed data, and everyone contributed to writing the manuscript.

Compliance with ethical standards

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed. This research was approved by the Animal Care and Use Committee (Protocol #04520-001) of Washington State University. Collections were approved by the Connecticut Department of Energy and Environmental Protection (Scientific collections permit #1115003) and Yale Myers Forest.

Conflict of interest The authors declare that they have no conflict of interest.

Data accessibility All data generated or analyzed during this study are included in this published article and its supplementary information files.

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